

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/36, 37/43	A1	(11) International Publication Number: WO 92/20371 (43) International Publication Date: 26 November 1992 (26.11.92)
(21) International Application Number: PCT/US92/03840 (22) International Filing Date: 7 May 1992 (07.05.92) (30) Priority data: 698,467 10 May 1991 (10.05.91) US (71) Applicant: CELTRIX PHARMACEUTICALS, INC. [US/ US]; 3055 Patrick Henry Drive, Santa Clara, CA 95052-8203 (US). (72) Inventors: BENTZ, Hanne ; 36125 Toulouse Street, Ne- wark, CA 94560 (US). ROSEN, David ; 3141 Valmaine Court, San Jose, CA 95135 (US). (74) Agents: CIOTTI, Thomas, E. et al.; Morrison & Foerster, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).		(81) Designated States: AU, CA, JP. Published <i>With international search report.</i>
(54) Title: TARGETED DELIVERY OF BONE GROWTH FACTORS (57) Abstract Methods and compositions for using conjugates of bone growth factors and targeting molecules in bone repair and augmentation are provided. The bone growth factor and targeting molecule are chemically conjugated to a crosslinker. The crosslinker is preferably a synthetic hydrophilic polymer. The bone growth factor is preferably TGF- β , activin, or bone morphogenic protein (BMP). Targeting molecules preferably have an affinity for bone, such as tetracycline, calcein, bisphosphonate, polyaspartic acid, polyglutamic acid, aminophosphosugars, or estrogen.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MI	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LJ	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

5

TARGETED DELIVERY OF BONE GROWTH FACTORSDescription10 Technical Field

This invention relates to the fields of protein engineering and medical treatment. More particularly, this invention relates to conjugates of bone growth factors and targeting molecules and their uses in bone repair and augmentation and the like.

Background of the Invention

"Transforming growth factor- β " (TGF- β) represents a family of proteins which are evolutionarily highly conserved, and which affect a broad spectrum of cell types. Two forms, TGF- β 1 and TGF- β 2, have been identified in platelet releasates and bone. Both proteins exist as 26 kD disulfide-linked homodimers. Even though there are 14 amino acid differences in the first 36 amino acids residues of the two forms, their biological activities are similar (Cheifetz et al., Cell (1987) 48:409-415; S. Seyedin et al., J. Biol. Chem. (1987) 262:1946-1949). Although TGF- β 1 and TGF- β 2 apparently bind to common cell surface receptors, the amino acid sequence homology is only about 70%. TGF- β 3 exhibits about 76% homology with TGF- β 1, and about 79% homology with TGF- β 2.

Additional TGF- β s have also been described. U.S. Patent No. 4,886,747 describes the identification of TGF- β 3 and its nucleotide sequence, and describes a

-2-

method for recovery of TGF- β 3 from recombinant cell cultures. S.B. Jakowlew et al., Molec. Endocrinol. (1988) 2:1186-1195 describes TGF- β 4 and its nucleotide sequence, identified by cDNA characterization. A.B.

- 5 Roberts et al., Growth Factors, Vol. 2 (1990), pp. 135-147, describes the purification of TGF- β 5 from Xenopus-conditioned medium.

TGF- β was first identified as a factor which permitted anchorage-independent growth of primary cell
10 cultures. In vivo, TGF- β promotes the deposition of connective tissue, and induces cell growth for cells of mesenchymal origin. TGF- β 1 and TGF- β 2 affect the proliferation and differentiation of cells of the immune system including macrophages (Wall et al., Proc. Natl.
15 Acad. Sci. U.S.A. (1987) 84:5788), pre-B cells (Lee et al., J. Exp. Med. (1987) 166:1290), hematopoietic stem cells (Ohta et al., Nature (1987) 329:539; Ishibashi et al., Blood (1987) 69:1737; Keller et al., J. Exp. Med. (1988) in press; Sing et al., Blood (1988) in press), and
20 NK cells (Rook et al., J. Immunol. (1986) 136:3916). Bentz et al., U.S. Patent No. 4,806,523, disclosed that TGF- β may also be administered to suppress inflammatory responses, including immune suppression. McPherson et al., U.S. Patent No. 4,816,442 disclosed that TGF- β may
25 also be administered to suppress hyperproliferation, for example, cancer and leukemia.

Activins are dimeric proteins structurally similar to inhibin, TGF- β 1, TGF- β 2, and other proteins that makeup a family of proteins structurally related to
30 TGF- β 1. These proteins exhibit the chromatographic properties of TGF- β s. In addition to having homology with respect to the amino acid sequences, activins exhibit conservation of cysteine positions characteristic of the TGF- β s. Activins exhibit a molecular weight of 25
35 kD under nonreducing conditions by SDS-PAGE (and a

-3-

molecular weight of 14 kD under reducing conditions). There are two known forms of the activin subunits, which have been termed β A or β B. Homodimeric forms β AA and β BB and a heterodimeric form β AB have been described in the literature. Activin subunits have about a 30% homology to TGF- β 1 and TGF- β 2 chains in terms of their amino acid sequences. Inhibins are polypeptides which are also structurally related to activins. Inhibins are heterodimers of the activin β A or β B subunit and a separate α subunit. Inhibins exhibit activity essentially opposite to activin.

The activin β A homodimer and β AB heterodimer have been purified from porcine follicular fluid, and have been shown to stimulate the release of follicle stimulating hormone (FSH) from rat pituitary cells *in vitro* (W. Vale et al., Nature (1986) 321:776-79). Other reported activities include stimulation of oxytocin release from neurosecretory neurons (P.E. Sawchemko, et al., Nature (1988) 334:615-17; W. Vale et al., "Recent Progress in Hormone Research" (1988) 44:1-34); stimulation of insulin secretion from pancreatic islets (Y. Totsuka et al., Biochem. & Biophys. Res. Comm. (1988) 156:335-39); and stimulation of erythroid and multi-potential progenitor cell colony formation in bone marrow culture (J. Yu et al., Nature (1987) 330:765-67; H.E. Broxmeyer et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85:9052-56). Activin β A is apparently identical to erythroid differentiation factor (EDF) (M. Murata et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85:2434-38).

Despite the fact that activin is similar in amino acid sequence to TGF- β , activin does not compete with TGF- β for binding to TGF- β receptors types I, II, or III present on fibroblasts and epithelial cells. However, activin has been reported to compete against binding of TGF- β 1 to rat pituitary tumor cells (S.

-4-

Cheifetz et al., J. Biol. Chem. (1988) 263:17225-28).
TGF- β 1 and TGF- β 2 have been reported to induce formation
of endochondral bone in vivo (M.E. Joyce et al., J. Cell
Biol. (1990) 110:2195-2207, H. Bentz, et al. (1989) J.
5 Biol. Chem., 264:20805-10).

A "bone morphogenetic protein" (BMP) was
extracted from demineralized bone using urea or guanidine
hydrochloride and reprecipitated according to the
disclosures in U.S. Patent Nos. 4,294,753 and 4,455,256
10 to Urist. Urist subsequently reported (Urist, M. R.,
Clin. Orthop. Rel. Res. (1982) 162:219) that ion exchange
purification of this crude protein mixture yielded an
activity which was unadsorbed to carboxymethyl cellulose
resin (CMC) at pH 4.8. Urist's reports in Science (1983)
15 220:680-685 and Proc. Natl. Acad. Sci. U.S.A. (1984)
81:371-375 describe BMPs having molecular weights of
17,500 and 18,500 daltons. Urist's patent publication,
EPA Publication No. 0212474, describes BMP fragments of
4,000 to 7,000 daltons obtained by limited proteolysis of
20 BMP.

U.S. Patent No. 4,608,199 describes a
bone-derived protein of 30,000-32,000 daltons. The
protein is described as being water soluble and having no
affinity for concanavalin A.

25 WO 88/00205 reports four proteins, designated
BMP-1, BMP-2 Class I ("BMP-2"), BMP-3, and BMP-2 Class II
("BMP-4"), that are alleged to have osteogenic activity.
It is not known whether these BMPs have osteogenic
activity by themselves or require combination with other
30 factors.

J.M. Wozney, in Growth Factor Research, Vol. 1
(1989), pp. 267-280, describes three additional BMP
proteins closely related to BMP-2, and which have been
designated BMP-5, BMP-6 and BMP-7.

35 WO 89/09787 and 89/09788 describe a protein

-5-

called "OP-1", now known to be BMP-7. The cloning of BMP-7 is described in E. Ozkaynak et al., EMBO Journal (1990) 9:2085-2093, and the purification of BMP-7 is described in T.K. Sampath et al., J. Biol. Chem. (1990) 265:13198-13205.

U.S. Patent No. 4,434,094 to Seyedin and Thomas reported the partial purification of a bone generation-stimulating, bone-derived protein by extraction with chaotropic agents, fractionation on anion and cation exchange columns, and recovery of the activity from a fraction adsorbed to CMC at pH 4.8. This new protein fraction was termed "osteogenic factor" (OF) and was characterized as having a molecular weight below about 30,000 daltons.

There are several references in the art to proteins modified by covalent conjugation to polymers, to alter the solubility, antigenicity and biological clearance of the protein. For example, U.S. Patent No. 4,261,973 disclosed the conjugation of several allergens to PEG (polyethylene glycol) or PPG (polypropylene glycol) to reduce the proteins' immunogenicity. U.S. Patent No. 4,301,144 disclosed the conjugation of hemoglobin with PEG and other polymers to increase the protein's oxygen carrying capability. EPO 98,110 disclosed coupling an enzyme or interferon to a polyoxyethylene-polyoxypropylene (POE-POP) block polymer increases the protein's halflife in serum. U.S. Patent No. 4,179,337 disclosed conjugating hydrophilic enzymes and insulin to PEG or PPG to reduce immunogenicity. Davis et al., Lancet (1981) 2:281-83 disclosed the enzyme uricase modified by conjugation with PEG to provide uric acid metabolism in serum having a long halflife and low immunogenicity. Nishida et al., J. Pharm. Pharmacol. (1984) 36:354-55 disclosed PEG-uricase conjugates administered orally to chickens, demonstrating decreased

-6-

serum levels of uric acid. Inada et al., Biochem. & Biophys. Res. Comm. (1984) 122:845-50 disclosed lipoprotein lipase conjugation with PEG to render it soluble in organic solvents. Takahashi et al., Biochem. & Biophys. Res. Comm. (1984) 121:261-65 disclosed HRP conjugated with PEG to render the enzyme soluble in benzene. Abuchowski et al., Cancer Biochem. Biophys. (1984) 7:175-86, disclosed that enzymes such as asparaginase, catalase, uricase, arginase, trypsin, superoxide dismutase, adenosine deaminase, phenylalanine ammonia-lyase, and the like, conjugated with PEG exhibit longer half-lives in serum and decreased immunogenicity. U.S. Patent No. 4,830,847, and European Patent Application Ser. No. 207,557 disclose diphosphonate-derivatized macromolecules which may be labeled with technium-99m.

Both antibodies and liposomes have been used to direct drugs to target sites (see, for example, Tyle and Ram, eds., Targeted Therapeutic Systems, Marcel Dekker, New York, 1990.)

Targeted delivery of bone growth factors may reduce harmful or undesirable effects of those molecules, allow the use of lower doses because relatively higher doses can be delivered to the site of interest, and may prolong the effect at the site of interest. In addition, the use of a targeting molecule that influences bone metabolism, including bone resorption and formation, may result in an additive or synergistic effect.

Summary of the Invention

It is an object of the invention to provide a composition comprising a bone growth factor and a targeting molecule having affinity for a tissue of interest, where the bone growth factor and targeting molecule are chemically conjugated to a crosslinker. The

-7-

crosslinker is preferably a synthetic hydrophilic polymer. The bone growth factor is preferably TGF- β , activin, or bone morphogenic protein (BMP). Targeting molecules preferably have an affinity for bone, such as

5 tetracycline, calcein, bisphosphonate, polyaspartic acid, polyglutamic acid, aminophosphosugars, or estrogen.

It is another object of the invention to provide a method for augmenting bone formation in a subject.

10 It is another object of the invention to provide a method for treating bone loss in a subject.

Modes of Carrying Out The Invention

15 A. Definitions

The term "bone growth factor" refers to the families of proteins which affect bone formation. Examples of bone growth factors include transforming growth factor-beta (TGF- β), activin, and bone morphogenic

20 factor (BMP).

The term "TGF- β " refers to beta-type transforming growth factors, including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, TGF- β 5, heterodimers of the TGF- β polypeptide chains (e.g. TGF- β 1.2), and fragments thereof, synthetic

25 peptides, and homologous proteins having substantially equivalent biological activity in any of the numerous known TGF- β assays, such as the one described in Methods for Preparation of Media, Supplements, and Substrate for Serum-free Animal Cell Culture (1984) pp. 181-194. Alan

30 R. Liss, Inc. This particular assay determines ability to induce anchorage-dependent growth in non-neoplastic normal rat kidney (NRK) fibroblasts by measuring the formation of cell colonies in soft agar. Other known TGF- β activity assays include but are not limited to

35 stimulation of osteoblast proliferation, inhibition of

-8-

keratinocyte proliferation, stimulation of collagen synthesis in a variety of cells, inhibition of mitogen-stimulated T-cell proliferation, and stimulation of chemotactic activity. Preparation of TGF- β 1 and TGF- β 2 is described in U.S. Patent No. 4,774,322, incorporated herein by reference. Additional TGF- β s have also been described. U.S. Patent No. 4,886,747 describes the identification of TGF- β 3 and its nucleotide sequence, and describes a method for recovery of TGF- β from recombinant cell cultures. S. B. Jakowlew et al., Molec. Endocrinol. (1988) 2:1186-1195, describes TGF- β 4 and its nucleotide sequence, identified by cDNA characterization. A.B. Roberts et al., Growth Factors, Vol. 2 (1990) pp. 135-147, describes the purification of TGF- β 5 from Xenopus-conditioned medium.

The term "TGF- β " as used herein is also intended to include the heterodimer TGF- β 2.3, disclosed in copending patent application U.S. Ser. No. 614,306, filed 11/16/90. TGF- β 2.3 may be prepared from an extract of bone by pooling side fractions from peaks of column chromatography, subjecting those fractions to reverse phase HPLC and recovering those fraction which migrate more slowly than TGF- β 2 by SDS-PAGE, subjecting those slower migrating fractions to FPLC and recovering those that migrate during a pH 4.6 to 6.7 gradient, subjecting the pH 4.6 to 6.7 eluant to reverse phase HPLC or gel electrophoresis, and recovering substantially pure TGF- β 2.3.

The term "TGF- β " as used herein is also intended to include any other synthetic molecule whose mechanism of action is mediated through the TGF- β receptor or second messenger pathway.

Because these proteins are non-species-specific in their activity they maybe used to treat subjects in general, including sport, pet, and farm animals, and

-9-

humans.

As used herein, "bone morphogenetic protein" (BMP) refers to a class of bone-derived proteins capable of inducing bone formation, as measured by activity in an in vivo rat bone formation assay. BMPs include BMP-1, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 and BMP-7, and fragments, deletions, additions, substitutions, mutations and modifications thereof which retain the biological characteristics of the natural BMP. The BMP may be of human, bovine, or other species origin.

The term "activin" as used herein refers to activin β AA, activin β AB, activin β BB, and fragments thereof, synthetic peptides, and proteins having similar activity in a standard cell culture assay where the protein stimulates the release of follicular stimulating hormone (FSH) from rat pituitary cells (Vale, et al., Nature (1986) 321:776). Briefly, in this assay, anterior pituitaries from adult male Sprague-Dawley rats (200-220 g) are dissociated by collagenase and plated at a concentration of 0.33×10^6 cells per well in 24 well tissue culture dishes. The cells are allowed to recover for 72 hr in medium containing 2% fetal bovine serum (FBS). Following the recovery period, the cells are washed twice in fresh medium containing 2% FBS. All treatments are added at this time and the cells are incubated for 72 hr. The media is then collected and the FSH levels are determined using a radioimmunoassay kit provided by the National Hormone and Pituitary program of NIADDK.

As used herein, the term "targeting molecule" refers to a molecule that binds to the tissue of interest or that influences metabolism of that tissue of interest. So, for example, bone-targeting molecules may include bone-seeking molecules such as tetracycline, calcein, bisphosphonates, chelators, phosphates, polyaspartic

-10-

acid, polyglutamic acid, aminophosphosugars, peptides known to be associated with the mineral phase of bone such as osteonectin, bone sialoprotein and osteopontin, proteins with bone mineral binding domains, and the like.

5 Bone-targeting molecules may also include molecules which affect bone resorption and bone formation rates, such as bisphosphonates, and estrogens and other steroids. These bone-targeting molecules may bring the bone growth factors to the bone and/or result in a synergistic or

10 additive effect on bone resorption or formation.

The term "hydrophilic polymer" as used herein refers to a synthetic or natural polymer having an average molecular weight and composition which renders the polymer essentially water-soluble. Most hydrophilic

15 polymers achieve this property by incorporating a sufficient number of oxygen (or less frequently nitrogen) atoms available for forming hydrogen bonds in aqueous solution. Hydrophilic polymers used herein will generally be propylene glycol, polyoxyethylene, poly-

20 ethylene glycol, polytrimethylene glycols, polylactic acid, or derivatives thereof. Other suitable polymers include polyoxyethylene-polyoxypropylene block polymers and copolymers. Naturally occurring polymers such as starch, heparin and the like are also included within the

25 scope of this invention. All suitable polymers will be non-toxic and non-inflammatory when administered subcutaneously, and will preferably be essentially non-degradable in vivo over a period of several months. Presently preferred hydrophilic polymers are synthetic,

30 preferably polyethylene glycols (PEG) having an average molecular weight between about 200 and about 10,000, more preferably between about 600 and about 8,000, and most preferably about 3400.

The PEG is preferably difunctional, i.e., a

35 crosslinker between the bone growth factor and the

-11-

targeting molecule, consisting of homobifunctional groups such as in PEG 600 diglycidylether, or of heterofunctional groups. Branched polyfunctional PEG crosslinkers may also be used. The nature of the functional groups on the crosslinker depends on the reactivities of the molecules to be conjugated.

The term "chemically conjugated" as used herein means attached through a covalent chemical bond. In the practice of the invention, a hydrophilic polymer and a bone growth factor may be chemically conjugated by using a linking radical, so that the polymer and the bone growth factor are each bound to the radical, but not directly to each other.

Those of ordinary skill in the art will appreciate that polymers such as polyethylene glycol cannot practically be prepared having exact molecular weights, and that the term "molecular weight" as used herein refers to the average molecular weight of a number of molecules in any given sample, as commonly used in the art. Thus, a sample of PEG 2000 might contain polymer molecules ranging in weight from, for example, 1,200 to 2,500 daltons. Specification of a molecular weight range indicates that the average molecular weight may be any value between the limits specified, and may include molecules outside those limits. Thus, a molecular weight range of about 800 to about 20,000 indicates an average molecular weight of at least about 800, ranging to about 20 kD.

The term "available lysine residue" as used herein refers to lysine side chains exposed on the outer surface of the bone growth factor or targeting molecule, which are positioned in a manner allowing reaction with activated PEG. In general, 10-100%, and more preferably, 10-50% of available lysine residues may be used. The number of available lysine residues may be determined by

-12-

reaction with sodium 2,4,6-trinitrobenzenesulfonate (TNBS). Bone growth factor genetic variants can be constructed so as to substitute lysine residues at specific sites in the molecule, thus improving the efficiency of the crosslinking. In practice, such substitution will be conservative so as not to interfere with the receptor binding of growth factor and will be within exterior or more hydrophilic regions of the growth factor.

10 The term "treat" or "treatment" as used herein refer to repair, prevention, or alleviation of bone defects, especially defects due to loss of bone. Treatment of bone defects includes augmentation or restoration of lost bone as well as the prevention of further bone loss due to metabolic disease, chronic inflammatory processes (e.g., osteoporosis, osteoarthritis, Paget's disease, osteomalacia, osteomalacia, multiple myeloma and other forms of cancer, and age-related loss of bone mass). Compositions of the invention may include additional biologically active factors to aid in healing or regrowth of normal tissue.

25 The term "tissue of interest" as used herein refers to a desired target in the body for treatment or for placement of the bone growth factor. Tissues of interest may include bone, cartilage, or other tissues or cell types to which bone growth factors may be targeted.

B. General Methods

30 The compositions of the invention comprise a bone growth factor chemically conjugated to a targeting molecule. Examples of chemistries for conjugating or coupling proteins have been summarized in the art (see, for example, P.Tyle and B. Ram, eds., Targeted Therapeutic Systems, Marcel Dekker, New York, 1990; and

-13-

Means and Feeney, "Chemical Modifications of Proteins, History and Applications," Bioconjugate Chem. 1:2-12 (1990), hereby incorporated by reference in their entirety).

5 One such method includes chemical conjugation of the bone growth factor to a selected synthetic hydrophilic polymer or polymers, which in turn is linked to a targeting molecule. Thus, for example, a reactive group on the bone growth factor may be conjugated through
10 a crosslinker to a polymer, and the targeting molecule may be conjugated to the bone growth factor-polymer through a second crosslinker, where the second crosslinker may be of the same or different species as the first, depending on the reactive groups involved in
15 the targeting molecule. Examples of reactive groups on proteins include but are not limited to the carboxyl groups of the C-terminus or of aspartic and glutamic acid; amino groups of N-terminal and lysine residues; imidazole of histidine and phenolic functions of
20 tyrosine; sulfhydryl groups of cysteine; and guanidine groups of arginine.

For example, TGF- β contains a number of available amino, carboxyl, and hydroxy groups which may be used to bind the synthetic hydrophilic polymer. The
25 polymer may be connected using a "linking group", as the native hydroxy or amino groups in TGF- β and in the polymer frequently require activation before they can be linked. One may thus employ compounds such as dicarboxylic anhydrides (e.g., glutaric or succinic anhydride)
30 to form a polymer derivative (e.g., succinate), which may then be activated by esterification with a convenient leaving group, for example, N-hydroxysuccinimide, N,N'-disuccinimidyl oxalate, N,N'-disuccinimidyl carbonate, and the like. Presently preferred dicarboxylic anhydrides that are used to form polymer-glutarate
35

-14-

compositions include glutaric anhydride, adipic anhydride, 1,8-naphthalene dicarboxylic anhydride, and 1,4,5,8-naphthalenetetracarboxylic dianhydride. The polymer thus activated is then allowed to react with the
5 TGF- β , forming a TGF- β -polymer conjugate.

TGF- β is difficult to dissolve in solutions of appropriate pH for coupling to hydrophilic polymers. In a presently preferred method, the TGF- β is lyophilized in the absence of a carrier protein, and dissolved in a mild
10 acid, preferably about 10 mM HCl. The solution is neutralized by adding a strong base, preferably NaOH (1 N solution) in buffered saline, additionally including a solubilizing amount of DMSO. The final solution preferably contains about 50% DMSO or CH₃CN to solubil-
15 ize the TGF- β and to prevent aggregation.

In one embodiment, monomethylpolyethylene glycol (mPEG) (mw 5,000) is reacted with glutaric anhydride to form mPEG glutarate. The glutarate derivative is then reacted with N-hydroxysuccinimide to
20 form a succinimidyl monomethylpolyethylene glycol glutarate. The succinimidyl ester is then capable of reacting with free amino groups present on a bone growth factor (lysine residues) to form a bone growth factor - PEG conjugate, which may then be further conjugated to a
25 targeting molecule. Other polymers may be substituted for the mPEG, as described above. Similarly, the coupling reaction may be carried out using any known method for derivatizing proteins and synthetic polymers.

The activated mPEG may be replaced, in whole or
30 in part, by bifunctional activated PEG (i.e., non-methylated PEG which is then activated at each end), thus providing a crosslinked or partially crosslinked bone growth factor composition. The character of the composition may be adjusted as desired, by varying the
35 amount of bifunctional PEG included during the process.

-15-

The resulting bone growth factor-targeting molecule conjugates may be purified by standard techniques, preferably by reverse-phase HPLC (RP-HPLC), size-exclusion HPLC (SEC-HPLC; e.g., tetrahydrogel-HPLC) or ion-exchange chromatography. RP-HPLC is preferably performed using a C18 column using 90% acetonitrile or 90% isopropanol with 0.1% trifluoroacetic acid (TFA) as the B solvent. For SEC-HPLC, a preferred running buffer can be 5 mM sodium acetate at pH 5.5.

Whether a homobifunctional group or a heterobifunctional group is used depends on the nature of the reactive groups on the bone growth factor and the targeting molecule. For example, homobifunctional reagents may have the general formula R-PEG-R, where R is glycidylether(e.g., to form diglycidyl-PEG 600), succinimidyl succinate, *p*-nitrophenylcarbonate (e.g., to form bis(*p*-nitrophenylcarbonate) PEG3400, imidazoylcarbonyl, and the like. These and similar crosslinkers are available from Sigma, Union Carbide, and Polyscience. Shorter crosslinkers such as carbodiimide, not containing the PEG bridging part, have well described chemistries in the art and are available commercially (e.g., Pierce).

The bone growth factor-targeting compositions of the invention are preferably administered by parenteral routes, intravenous injection, intranasal or bronchial aerosol, and the like. Sustained release devices may be surgically implanted under the skin or within the peritoneal cavity.

Pharmaceutical formulations of the invention which include a bone growth factor and a targeting molecule for administration will generally include an osteogenically effective amount of the bone growth factor to promote bone growth, in addition to a pharmaceutically acceptable excipient. Suitable excipients

-16-

include most carriers approved for parenteral administration, including water, saline, Ringer's solution, Hank's solution, and solutions of glucose, lactose, dextrose, ethanol, glycerol, albumin, and the like.

5 These compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and other accessory additives. The bone growth factor-targeting composition may also be delivered in a slow release form from a suitable carrier.

10 A presently preferred vehicle comprises about 1 mg/ml serum albumin (species-specific) in phosphate-buffered saline (PBS) or isotonic citrate buffer. A thorough discussion of suitable vehicles for parenteral administration may be found in E.W. Martin, "Remington's
15 Pharmaceutical Sciences" (Mack Pub. Co., current edition sections relating to the excipient vehicles and formulating being incorporated herein by reference to disclose such). Such formulations are generally known to those skilled in the art and are administered systemically to
20 provide systemic treatment.

The "effective amount" of bone growth factor - targeting composition necessary to effect treatment will depend upon the growth factor, the nature and severity of the condition to be treated, the age and general health
25 of the subject, the specific activity of the composition prepared, and other factors which may be determined by the practitioner of ordinary skill in the art. As a general guide, however, an effective amount of bone growth factor-targeting molecule conjugate for treating a
30 bone loss or defect will range from about 0.1 $\mu\text{g/kg}$ to about 25 $\mu\text{g/kg}$.

The bone growth factor - targeting compositions of the invention may be formulated as solutions or suspensions, or may be lyophilized for later
35 reconstitution.

-17-

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(TGF- β -PEG-Tetracycline Conjugate Preparation)

10 Tetracycline (2 μ mol; Aldrich Chem. Co.) is dissolved in 1 μ mol bis-epoxy-PEG (Polyscience) and reacted at 90°C under nitrogen for 24 hr with gentle stirring. The resulting tetracycline-PEG conjugate is then reacted with TGF- β 2 by adding 100 μ g TGF- β 2
15 dissolved in 0.02 M sodium borate, 0.02% SDS, 50% acetonitrile, pH 9.0 to 52 μ l of the tetracycline-PEG conjugate. The mixture is incubated at 37° C for 48 hr. The resulting TGF- β -PEG-tetracycline conjugates are then purified by molecular sieve chromatography and C18-RP-
20 HPLC.

Example II

(Conjugation of TGF- β 2 to 3-amino-1-hydroxypropylidene-1,1-bisphosphonates (ADP) via bis-epoxy PEG 600).

25 Lyophilized TGF- β is dissolved in 50% water/acetonitrile (final concentration 200-500 μ g/ml). The pH is adjusted to 7-8 with phosphate or borate buffer. A 1-20 molar excess of bis-epoxy-PEG 600 or tetra-epoxy PEG 1700 (Polyscience) is added and the
30 mixture is reacted for 15-20 hours at pH 7.0-8.0 at 40°C. Amicon ultrafiltration may be used to remove unattached PEG. The pH is raised to 9.5 with 1% deoxycholate, pH 9.5, 0.01 N NaOH, or 0.1 M borate buffer. The 3-amino 1-hydroxypropylidene 1,1-bisphosphonate (APD, CIBA-GEIGY)
35 is dissolved in 1% deoxycholate, pH 9.5, at 2 mg/ml. The

-18-

bisphosphonate is then added in a 5-200 molar excess and stirred overnight at 40°C. The reaction mixture is acidified and filtered through a 0.45 μ m low protein binding syringe filter prior to chromatography.

- 5 Purification is performed by gel filtration, ion exchange (Mono-S) and reverse-phase HPLC.

Example III

(Conjugation of TGF- β to 3-amino-1-hydroxypropylidene-
10 1,1-bisphosphonate (APD) carbodiimide)

Lyophilized TGF- β (200 μ g) is dissolved in 0.1 M MES (2-(N-Morpholino) ethanesulfonic acid) buffer, 50% acetonitrile, at pH 4.7. Sulfo-NHS (N-hydroxysulfosuccinimide, Pierce) is dissolved in aqueous
15 50% acetonitrile and 2-10 μ moles are added to the TGF- β solution. The water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCL (EDC, Pierce) is dissolved in 0.1 MES buffer, pH 4.7, and added in 2-50 molar amounts. Finally, the bisphosphonate (ADP, CIBA-
20 GEIGY) dissolved in 0.1 N HCL (5 mg/100 μ l) is added in 2-50 μ molar amounts. The pH is adjusted to 4.7-5.5 with 0.01 N NaOH. The mixture is reacted overnight at room temperature. The TGF- β /APD conjugate is purified by ion-exchange (Mono-S) chromatography and reverse-phase HPLC.

25

Example IV

(Conjugation of TGF- β to Tetracycline (Einhorn Reaction))

Tetracycline dissolved in methanol is reacted with 38% formaldehyde. TGF- β dissolved in a 1:5 solution
30 of water:methanol is added dropwise. The reaction mixture is stirred gently for 2 hr at room temperature. The resulting TGF- β -N-lysinemethyltetracycline can be separated from other reaction products by size-exclusion and RP-HPLC. (Bernardelli, et al. Liebigs Ann. Chem.
35 706:243-249 (1967)).

-19-

Example V(Conjugation of TGF- β to Tetracycline)

Alkoxyalkyltetracyclines can be obtained by
5 condensing a tetracycline with an aldehyde in alcohol.
For example, tetracycline, mono-hydroxy-methoxy PEG-2000
(MeO-PEG 2000-OH), and formalin are added in equimolar
amounts to a round bottom flask fitted with a reflux
condenser. The mixture is dissolved in tert butyl
10 alcohol by heating to approximately 80°C (oil bath). The
temperature is increased to 115°C and held for 50 hours.
This reaction is conducted under nitrogen. The system is
then attached to a water aspirator to remove water from
the reaction mixture, after which the mixture is allowed
15 to cool to room temperature. Ethanol is added to
dissolve the tert butyl alcohol and the mixture is
filtered. The remaining wine-red precipitate is further
purified by acetone extraction. The so-obtained
intermediate can be further purified by ion exchange and
20 size exclusion chromatography. After activation, the
tetracycline-PEG conjugate can then be coupled to the
TGF- β , preferably in an organic solvent such as methylene
chloride, dimethylformamide, DMSO, and the like.

25

30

35

-20-

WHAT IS CLAIMED:

1. A composition comprising a bone growth factor and a targeting molecule having affinity for a tissue of interest, wherein said bone growth factor and targeting molecule are chemically conjugated to a crosslinker.
2. The composition of claim 1, wherein the bone growth factor is TGF- β .
3. The composition of claim 1, wherein the bone growth factor is an activin.
4. The composition of claim 1, wherein the bone growth factor is a bone morphogenic protein (BMP).
5. The composition of claim 1, wherein the crosslinker is a synthetic hydrophilic polymer.
6. The composition of claim 1, wherein the crosslinker is polyfunctional.
7. The composition of claim 5, wherein said polymer is polyethylene glycol.
8. The composition of claim 7, wherein said polyethylene glycol has a molecular weight of about 200 to about 10,000.
9. The composition of claim 5, wherein said synthetic hydrophilic polymer is bound to an available lysine residue on said bone growth factor.

-21-

10. The composition of claim 5, wherein said growth factor has a plurality of available lysine residues and molecules of said synthetic hydrophilic polymer are bound to 10-100% of said available lysine residues.

11. The composition of claim 5, wherein said growth factor has a plurality of available lysine residues and molecules of said synthetic hydrophilic polymer are bound to 10-50% of said available lysine residues.

12. The composition of claim 1, wherein said targeting molecule has an affinity for bone.

13. The composition of claim 1, wherein said targeting molecule is a protein.

14. The composition of claim 1, wherein said targeting molecule is tetracycline.

15. The composition of claim 1, wherein said targeting molecule is calcein.

16. The composition of claim 1, wherein said targeting molecule is a bisphosphonate.

17. The composition of claim 1, wherein said targeting molecule is polyaspartic acid.

18. The composition of claim 1, wherein said targeting molecule is polyglutamic acid.

19. The targeting molecule of claim 1, wherein said targeting molecule is an aminophosphosugar.

-22-

20. The targeting molecule of claim 1, wherein said targeting molecule is a steroid.

5 21. The targeting molecule of claim 1, wherein said targeting molecule is an estrogen.

22. A method for augmenting the formation of mature bone comprising administering an effective amount
10 of the composition of claim 1 in a pharmaceutically acceptable carrier.

23. A method for treating bone loss in a subject, comprising administering the composition of
15 claim 1 to the subject.

24. The method of claim 23, wherein said bone growth factor is TGF- β .

20 25. The method of claim 23, wherein said bone growth factor is an activin.

26. The method of claim 23, wherein said bone growth factor is a bone morphogenic protein (BMP).

25 27. The composition of claim 23, wherein said targeting molecule has an affinity for bone.

28. The method of claim 23, wherein said
30 targeting molecule is tetracycline.

29. The method of claim 23, wherein said targeting molecule is a calcein.

-23-

30. The method of claim 23, wherein said targeting molecule is a bisphosphonate.

31. The method of claim 23, wherein said
5 targeting molecule is polyaspartic acid.

32. The method of claim 23, wherein said targeting molecule is polyglutamic acid.

10 33. The method of claim 23, wherein said targeting molecule is an aminophosphosugar.

34. The method of claim 23, wherein said targeting molecule is an estrogen.

15 35. The method of claim 23, wherein the subject is human.

36. The method of claim 23, wherein the bone
20 loss is osteoporosis-related.

37. The method of claim 23, wherein the bone loss is osteomalacia-related.

25 38. The method of claim 23, wherein the bone loss is osteomalacia-related.

39. The method of claim 23, wherein the bone loss is age-related.

30 40. The method of claim 23, wherein the bone loss is related to steroid therapy.

41. The method of claim 23, wherein the bone
35 loss is osteoarthritis-related.

-24-

42. The method of claim 23, wherein the bone loss is related to Paget's disease.

5 43. The method of claim 23, wherein the bone loss is related to cancer.

 44. The method of claim 40, wherein the cancer in multiple myeloma.

10

 45. The method of claim 23, wherein the treatment is prophylactic.

 46. The method of claim 23, wherein the composition contains a sustained-release vehicle.

15

20

25

30

35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03840

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/36, 37/43

US CL :514/12, 21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
514/2, 8, 12, 21 US and foreign documentationElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS TGF, Osteoporosis, Bone Growth Factor, Conjugated, Diphosphonate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E,&	US, A, 5,118,667 (Adams et al) 02 June 1992, see the entire document.	1-46
A	US, A, 4,863,899 (Todaro) 05 September 1989, see the entire document.	1-46

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 08 AUGUST 1992	Date of mailing of the international search report 19 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer FATEMEH T. MOEZIE
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-4200